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Work fluctuations, transient violations of the second law and free-energy recovery methods: Perspectives in Theory and Experiments

Félix RITORT
Departament of Physics
Faculty of Physics
University of Barcelona,
Diagonal 647
08028 Barcelona, Spain
and
Department of Physics
University of California
Berkeley CA 94720, USA

Abstract. In this report I discuss fluctuation theorems and transient violations of the second law of thermodynamics in small systems. Special emphasis is placed on free-energy recovery methods in the framework of non-equilibrium single molecule pulling experiments. The treatment is done from a unified theoretical-experimental perspective and emphasizes how these experiments contribute to our understanding of the thermodynamic behavior of small systems.

1 Biophysics and statistical physics

Living systems are the most notable example of how matter can organize into states of extremely high complexity. The investigation of the structural organization of biological matter was boosted since the discoveries of the double helix structure of the DNA by Watson and Crick and the ensuing discovery of the structure of various proteins half century ago. Microscopic and spectroscopic techniques have greatly developed since then and current research is revealing an unprecedented richness of details about the functional behavior of living systems at the molecular and cellular level.

Biophysics is an area of science at the interface of physics, chemistry and biology. It is probably the most important interdisciplinary area of research whose knowledge requires a good understanding of how matter functions at the physical, chemical or biological level. While physics during the past has traditionally avoided the study of complex systems as imperfect, unapproachable or even uninteresting, the fact is that complexity is becoming a more and more common abode for physicists [1]. Among all possible disciplines in physics, statistical physics occupies a

privileged position as the natural framework to understand the behavior of biological systems at the molecular level. Stochasticity, fluctuations, metastability and thermal activation are concepts that are commonly used in statistical physics, yet they are also relevant to understanding the great variety of tasks carried out by biomolecules.

Thermodynamics is the discipline that describes the exchange processes of energy and matter that occur at the molecular and cellular level. However, thermodynamics, a science inherited in the 18th century from the times of the industrial revolution, has been inspired by motors and steam engines that proved to be indispensable during that time. It is fair then to question the relevance and applicability of all this knowledge when scientists immerse into the realm of the very small, far from the initial context that inspired Carnot and others. There has been a recent interest in the study of the so called work fluctuations and transient violations of the second law in systems driven to a non-equilibrium state. Fluctuation theorems quantify the probability of those non-equilibrium trajectories that, taken individually, violate some of the inequalities of thermodynamics. For macroscopic systems these trajectories are known to be irrelevant and unobservable, however at the level of the small, when the energies interested are of order of several times $k_B T$, these rare trajectories might become important. Although thermodynamic inequalities are known to describe the behavior of average values, it is important to explore the implications and relevance of these deviations in our understanding of energy transformation processes at the molecular level. A quantitative experimental observation and measurement of these trajectories has only recently become possible. This report describes these experiments from a unified theoretical-experimental perspective and emphasizes how these experiments contribute to our understanding of the thermodynamic behavior of small systems.

Sec. 2 is a short reminder about the second law of thermodynamics. It serves to explain the importance of fluctuations in small systems and short times. Section 3 describes work fluctuations in the framework of stochastic systems. Particular emphasis is put in the case where the system, initially in an equilibrium state, is perturbed arbitrarily far from equilibrium. We then discuss the non-equilibrium work relation originally derived by Jarzynski. Sec. 4 describes the current state of the art regarding experimental measurements of work fluctuations. Sec. 5 presents a digression on single molecule experiments as an excellent framework to investigate work fluctuations and free-energy recovery methods applied to biomolecules. Sec. 6 describes some of the experiments conducted in the unfolding of small RNA molecules under the action of an external force and the test of the Jarzynski equality. Sec. 7 illustrates a model where work fluctuations can be analytically computed as well as a comparison with the experiments reported in the preceding section. Finally, Sec. 8 presents some conclusions and perspectives.

2   Few facts about the second law of thermodynamics

To put in perspective the content of the present article we start by recalling few facts about the second law of thermodynamics [2, 3]. Let us consider a gas consisting of N molecules enclosed in a given vessel of volume V. The vessel is in contact with a thermal bath at temperature T and the gas inside is kept in equilibrium (i.e. its macroscopic properties remain stationary), see Fig. 1. Particles in the gas collide with the walls of the container exerting a pressure P that is function of the volume V and the temperature T. Their relation defines the equation of state of the gas. In these conditions heat is continuously exchanged between the gas and the bath through the walls of the vessel. The state of the gas can be modified by changing (e.g. expanding) the volume of the container from an initial volume V_i to a final volume V_f. If the transformation is done by keeping the system always in contact with the bath at temperature T the process is called isothermal. If the transformation is done slow enough then the gas goes through a sequence of equilibrium states and the process is called reversible. In general the transformation will not be reversible and the gas will be driven to a non-equilibrium state after the volume has been expanded. For the transformation $V_i \to V_f$ the first law of thermodynamics states that energy is conserved,

$$\Delta E = \Delta Q + W = \Delta Q + P\Delta V \tag{1}$$

with $\Delta V = V_f - V_i$. From (1) we see that the variation of the energy of the gas ΔE is the sum of the work exerted upon the system W plus the net heat supplied from the bath to the system ΔQ. The difference between heat and the other state variables E and V is important. If the volume or energy characterize the thermodynamic state of the system, the amount of heat contained does not as it is fully interchangeable with work depending on the path followed during the transformation. In a general transformation, part of the total work exerted upon the system is lost and dissipated in the form of heat to the surroundings. This is the content of the second law as stated by Clausius,

$$\Delta Q \leq T\Delta S \tag{2}$$

where $S(V,T)$ is a state function called entropy. The amount of heat lost during the process is called dissipated work W_{dis}. It is given by the difference between the maximum amount of heat that can be supplied to the system and the actual heat supplied (i.e. the right and left hand sides of (2)),

$$W_{\mathrm{dis}} = T\Delta S - \Delta Q \quad \text{with} \quad W_{\mathrm{dis}} \geq 0 \quad . \tag{3}$$

Another way to state the content of the second law is in terms of the Helmholtz free energy $F = E - TS$. Using (1,2,3) we have,

$$\Delta F = \Delta E - T\Delta S = \Delta Q + W - T\Delta S = W - W_{\mathrm{dis}} = W_{\mathrm{rev}} \tag{4}$$

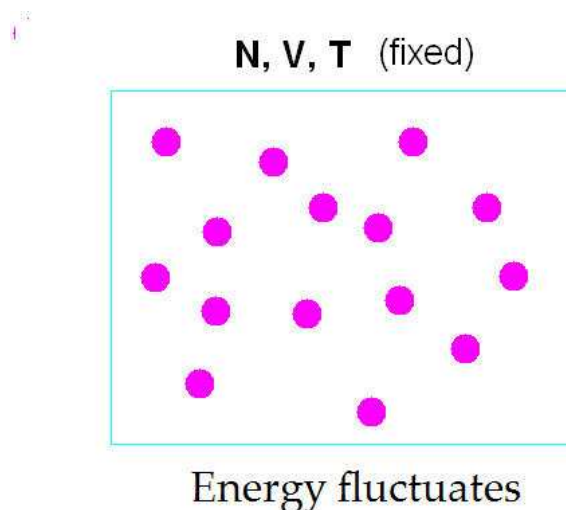**N, V, T** (fixed)

Energy fluctuates

Figure 1: Schematic representation of a vessel containing molecules that exchange energy and momentum with the surrounding bath through different mechanisms such as collisions with the walls. If the number of particles is small pressure fluctuations (due to the fluctuations in the collision rate of the molecules against the walls) could be observable with highly sensitive instruments.

where $W_{\rm rev}$ is the so-called reversible work, identical to the free energy change ΔF associated to the initial and final equilibrium states. Only in a reversible transformation the equality (2) is satisfied and $W = W_{\rm rev}$ or $W_{\rm dis} = 0$.

It has been known since the early days of statistical mechanics that the work can fluctuate [1]. To better clarify what we mean by this let us go back to the previous example and consider the gas of molecules enclosed in the vessel depicted in Fig. 1. Let us imagine that we repeat many times the experiment of the expansion of the container from V_i to V_f by following always the same variation protocol $V(t)$ where t is the time and the whole expansion lasts for a time t_0 [2]. Then for each experiment a different work value $W = \int PdV$ would be obtained as the pressure itself is a fluctuating variable. For a macroscopic system pressure fluctuations are unobservable due to the large number of molecules (of the order of the Avogadro number $N_A \sim 10^{23}$). However, for small systems, fluctuations could be observable. The result that intensive quantities display thermally induced fluctuations was put forward by Niquist for the case of voltage fluctuations across

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[1] The existence of rare fluctuations have given rise to several paradoxes, see [4] for an historical perspective

[2] The simplest protocol would be an expansion of the volume at a constant rate r, $\dot{V} = r = \frac{\Delta V}{t_0}$ or $V(t) = V_i + rt$

a resistance [5] and generalized later on by Welton and Callen [6]. Let us focus on the case where the gas contains about 100 molecules and is kept in equilibrium inside a volume V at the room temperature $T = 298K$. Let us imagine that at every interval of time (e.g. $\tau = 1$ millisecond) we count the number of collisions of the molecules against the wall, $N_c(\tau)$, as well as the average momentum $< p_c >$ transferred by the colliding molecules to the walls along each time interval. Then, the pressure exerted by the molecules on the walls P will be proportional to $N_c(\tau)$ and $< p_c >$. An histogram of the values of P thereby collected would show a Gaussian distribution centered around a mean value P_{mean} (which would also coincide with the most probable value) and a variance that decreases like $1/N$. This is the content of the law of large numbers. Sometimes the measured pressure will be large, sometimes it will be small: the fluctuations will become observable as the number of molecules is reduced. Initially these fluctuations will show a characteristic Gaussian profile. However, upon reduction of the number of molecules, deviations from the Gaussian behavior will be observed. The same happens in the non-equilibrium experiment where the volume is expanded. If the time t_0 is short enough or the number of molecules N is small enough, the work $W = \int PdV$ exerted upon the system will fluctuate from one non-equilibrium trajectory to another, and the fluctuations will become more noticeable as t_0 and N decrease. If the gas is initially in an equilibrium state, then (2,3) holds in average. However, for some trajectories the work will be such that $W_{\text{dis}} < 0$ and the inequality (2,3) will be reversed. These particular set of trajectories are called violating trajectories or transient violations of the second law.

The theory describing thermal fluctuations in equilibrium systems was put forward long ago by Einstein. However, this theory describes fluctuations of extensive quantities (such as the energy) in different ensembles in the macroscopic regime where fluctuations are subleading of order $\mathcal{O}(\sqrt{V})$, yet large. The relative magnitude of these fluctuations compared to the actual value of the energy content is of order $\mathcal{O}(1/\sqrt{V})$. In macroscopic samples relative fluctuations are of order $\mathcal{O}(1/\sqrt{N_A})$. This gives estimates for the magnitude of relative fluctuations of the order of 10^{-11}. A theory of fluctuations for small systems (such as those relevant to biophysics) where relative fluctuations are much larger (e.g. of order one) should be explored if the theory of fluctuations, as has been developed for macroscopic systems, reveals inadequate to explain the behavior observed in future experiments.

3 Fluctuation theorems in non-equilibrium systems

As we discussed in the previous section the work is quantity that fluctuates among different repetitions of the same experiment. Moreover, as compared to other quantities such as the internal energy, the entropy or the heat transferred, the amount of work exerted upon the system is a directly measurable quantity. A relevant question is then to ask what can we learn by measuring work fluctuations.

To answer this question we consider the dynamical evolution of a system in contact with a large bath or reservoir where heat and/or matter can be continuously exchanged. Both system and bath may appear inextricably linked and no partial description of the system can be achieved without considering the behavior of the bath and its interaction with the system. The mathematical treatment of the combined system plus bath complex represents a formidable theoretical challenge and some coarse-graining strategies must be considered to tackle this question. A common strategy is to consider a reduced level in the description of the dynamics of the system where many details of the bath have been eliminated in favor of a few number of parameters (such as its temperature, its pressure or its chemical potential). These parameters are thought to be sufficient to characterize the heat and/or matter exchange between system and bath. After this reduction is adopted, few requirements have to be imposed on the dynamics in order to reproduce many of the observable properties. In particular, dynamics must be microscopically reversible (or satisfying detailed balance) and ergodic (all configurations must be accessible) [3] to guarantee that the system reaches thermal equilibrium after long times so the net heat exchange between system and bath asymptotically decays to zero.

3.1   Work fluctuations in stochastic systems

It is in the framework of such coarse-grained dynamics that we want to focus our discussion and investigate the origin of work fluctuations. A prominent example of such reduced dynamics are stochastic Markov processes. The content of this section will be useful to establish the notation that we will use throughout the paper. We now deepen the mathematical level of our discussion and consider a general system described by an energy function $E(\mathcal{C})$ where $\mathcal{C}$ is a generic configuration (in the example in Sec. 2 of a gas of N molecules, $\mathcal{C}$ would stand for the positions and momenta of all molecules inside the vessel) in contact with a bath at temperature T. The dynamics are assumed to be discrete in time with elementary step Δt. A trajectory of the system is characterized by the sequence of configurations $\mathcal{T} \equiv \{\mathcal{C}_k; 0 \le k \le N_s\}$ where k is the index for the discrete time step and N_s is the total number of time steps. The time corresponding to step k is then given by $t = k\Delta t$ with $t = 0 (k = 0)$ and $t_f(k = N_s/\Delta t)$ denoting the initial and final times respectively. The continuous-time limit is recovered if $\Delta t \to 0, N_s \to \infty$ with t_f finite. Dynamics are then defined by the set of probabilities $P_k(\mathcal{C})$ for the system to be found at configuration $\mathcal{C}$ at time-step k. The $P_k(\mathcal{C})$ satisfy a master equation. For a Markov process the time evolution of these probabilities depends upon the form of the rates $W_k(\mathcal{C}'|\mathcal{C})$, defined as the transition probability per unit time to go from configuration $\mathcal{C}$ to $\mathcal{C}'$ at time-step k. These rates are assumed to lead to an ergodic dynamics (where any pair of configurations are always connected by at

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[3]Ergodicity is not an essential property if one considers equilibrium restricted to a given region of phase space, the sole condition is that all configurations contained in that region of phase space must be accessible during the dynamical process.

least one trajectory) and satisfy the detailed balance condition,

$$\frac{W_k(\mathcal{C}'|\mathcal{C})}{W_k(\mathcal{C}|\mathcal{C}')} = \exp\Big(-\beta(E(\mathcal{C}') - E(\mathcal{C}))\Big) \tag{5}$$

where $\beta = 1/k_B T$, k_B being the Boltzmann constant. Under very general conditions this dynamics guarantees that the system reaches a stationary state where configurations are populated according to the Boltzmann weight. The solution to the master equation gives the time evolution for the system.

Now we will treat the case where the system is perturbed in a prescribed way and consider the ensemble of all possible non-equilibrium trajectories that start from an initial state characterized by the distribution $P_0(\mathcal{C})$. Because dynamics is stochastic, it will generate an ensemble of non-equilibrium trajectories by repeating the same experiment many times [4]. In addition to the configuration $\mathcal{C}$, and in order to characterize the perturbation protocol, we introduce a parameter λ that specifies the value of the control parameter that is changed throughout the non-equilibrium process [5]. An important remark is now in place. The control parameter is a variable that can change in time but does not fluctuate. The temporal sequence of values $\{\lambda_k; 0 \leq k \leq N_s\}$ defines the perturbation protocol and this sequence of values never changes from experiment to experiment. Somehow, the control parameter plays a role akin to the temperature of the bath. In particular, for a fixed value of λ, we require that dynamics is such that the system asymptotically reaches the thermodynamic state corresponding to that value of λ. To understand how rates depend on the value of λ we reason as follows. The control parameter usually shifts the energy levels of the system according to the relation,

$$E_\lambda(\mathcal{C}) = E(\mathcal{C}) - \lambda A(\mathcal{C}) \tag{6}$$

where $A(\mathcal{C})$ is the observable coupled to the parameter λ [6]. The simplest assumption is then to enforce detailed balance for the perturbed rates,

$$\frac{W_\lambda(\mathcal{C}'|\mathcal{C})}{W_\lambda(\mathcal{C}|\mathcal{C}')} = \exp\Big(-\beta(E_\lambda(\mathcal{C}') - E_\lambda(\mathcal{C}))\Big) = \frac{W(\mathcal{C}'|\mathcal{C})}{W(\mathcal{C}|\mathcal{C}')} \exp\big(-\beta\lambda\Delta A\big) \tag{7}$$

[4] The same result holds for deterministic (e.g. Hamiltonian) dynamics. In this case the ensemble of non-equilibrium trajectories is determined by the ensemble of initial configurations sampled with probability $P_0(\mathcal{C})$. The set of phase space points then behaves as an incompressible fluid, a consequence of the Liouville theorem. Hamiltonian dynamics can be seen as a particular limit of stochastic dynamics, where rates $W_k(\mathcal{C}'|\mathcal{C})$ vanish except along the constant energy surface $E(\mathcal{C}) = E(\mathcal{C}')$ and are deterministic, i.e. rates are different from zero only for pairs of configurations $\mathcal{C}, \mathcal{C}'$ connected by the equations of motion. Dynamics is reversible and corresponds to (5) with $W_k(\mathcal{C}'|\mathcal{C}) = W_k(\mathcal{C}|\mathcal{C}')$. The case of Hamiltonian dynamics was originally addressed by Jarzynski in his original derivation of the non-equilibrium work relation [7]. The stochastic case has been analyzed also for general Markov processes by Crooks and Jarzynski [8, 9, 10] and for Langevin dynamics by Kurchan [11]. For a discussion of the similarities and differences between deterministic and stochastic dynamics see [12].

[5] For simplicity we only consider the case of one control parameter. For many control parameters the generalization is straightforward.

[6] For instance, if λ is a magnetic or gravitational field then A stands for the magnetization and the height of the center of mass respectively

where we used (5) in the r.h.s. and the definition $\Delta A = A(\mathcal{C}') - A(\mathcal{C})$. We now consider the variation of energy along a given trajectory $\Delta E(\mathcal{T}) = E_{\lambda_f}(\mathcal{C}_f) - E_{\lambda_0}(\mathcal{C}_0)$ where $\mathcal{C}_I, \mathcal{C}_f$ are the initial and final configurations for that trajectory and λ_0, λ_f are the initial and final values of the control parameter as defined by the protocol (trajectory independent). From (6) this is given by,

$$\Delta E(\mathcal{T}) = \Big[\sum_{k=0}^{N_s-1} (E_{\lambda_k}(\mathcal{C}_{k+1}) - E_{\lambda_k}(\mathcal{C}_k)) \Big] - \Big[\sum_{k=0}^{N_s-1} A(\mathcal{C}_k)\Delta\lambda_k \Big] = \Delta Q(\mathcal{T}) + W(\mathcal{T}) \quad (8)$$

with $\Delta\lambda_k = \lambda_{k+1} - \lambda_k$. This decomposition was proposed originally by Crooks [9] to identify work and heat by using the first law of thermodynamics (1). The first term in (8) is identified as the heat transferred from the bath to the system and the second with the work exerted upon the system. We concentrate our attention on the the work exerted upon the system along a given trajectory $\mathcal{T}$,

$$W(\mathcal{T}) = \sum_{k=0}^{N_s-1} \Big(\frac{\partial E_\lambda(\mathcal{C}_k)}{\partial\lambda} \Big)_{\lambda=\lambda_k} \Delta\lambda_k = -\sum_{k=0}^{N_s-1} A(\mathcal{C}_k)\Delta\lambda_k \equiv -\int_0^t ds\,\dot\lambda(s)A(\mathcal{C}(s))ds$$

$$(9)$$

where we have applied the continuous-time limit [7] in the last term in the r.h.s. of (9). As the trajectory is stochastic the work is a fluctuating quantity that can be characterized by its probability distribution $\mathcal{P}(W)$ defined as,

$$\mathcal{P}(W) = \sum_{\mathcal{T}} P(\mathcal{T})\delta(W - W(\mathcal{T})) \qquad (10)$$

where $\mathcal{T}$ stands for the trajectory and was already defined. The importance of $\mathcal{P}(W)$ relies upon the fact that it is a quantity that is experimentally measurable and therefore is suitable to quantitatively characterize work fluctuations along non-equilibrium trajectories.

3.2   The fluctuation theorem (FT)

Fluctuation theorems (FTs) provide specific relations for the quantity $\mathcal{P}(W)$ in (10) for general non-equilibrium processes. In fact, until now nothing was said about the type of non-equilibrium process and the treatment given in the previous section was general. We defined concepts such as the initial and final state, the perturbation protocol $\lambda(t)$, the trajectory $\mathcal{T}$ and the work and heat along a given trajectory. The main difference between a general non-equilibrium process and a reversible one is the enormous and various type of situations one can encounter in the first case. General physically meaningful statements about the properties of the distribution (10) quite probably do not exist and a specific type of non-equilibrium

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[7]In the continuous-time limit, both $\{\mathcal{C}_k, \lambda_k\}$ become $\mathcal{C}(t), \lambda(t)$ defining the real-time trajectory and perturbation protocol respectively.

process has to be adopted to come up with specific results. Several fluctuation theorems have appeared in the literature depending on the particular non-equilibrium context. Many fall into the category of entropy production FTs. The first example in this class was proposed by Evans, Cohen and Morriss [13] for systems in steady states. The entropy production there defined bears some resemblance with the work (9) that is exerted by the external non-conservative forces that act upon the system. Several related theoretical results have followed [14, 15, 16] as well as experiments [17, 18]. A comprehensive review can be found in [19]. Other more complex scenarios can be envisaged, for example in the case where the system is in a non-stationary aging state[8]. In this case, no work is performed upon the system and the relevant quantity turns out to be the released heat from the system to the bath [22, 23].

The content of this article deals particularly with systems initially in equilibrium with the bath that are driven to a non-equilibrium state by the action of an applied perturbation [20]. Therefore,

$$P_0(\mathcal{C}) = P_{\text{eq}}(\mathcal{C}) = \frac{\exp(-\beta E_{\lambda_0}(\mathcal{C})}{Z} \tag{11}$$

where $Z = \sum_{\mathcal{C}} \exp(-\beta E_{\lambda_0}(\mathcal{C}))$. This case has been studied by Jarzynski [7, 8] and Crooks [9, 10]. We omit details of the derivation as these can be found in the references. A particularly interesting identity has been derived by Crooks [10] who considered the forward and reverse paths in a non-equilibrium process. The forward process (F) is characterized by the protocol function $\lambda_F(t)$ with the initial state in equilibrium at the value $\lambda_F(0)$. The reverse process (R) is characterized by the inverted protocol function $\lambda_R(t) = \lambda_F(t_0 - t)$ with t_0 being the total time for the forward process and the initial state for the reverse process being in equilibrium at the value $\lambda_R(0)$ (which is equal to $\lambda_F(t_0)$). The following result is obtained [10],

$$\frac{P_F(W)}{P_R(-W)} = \exp\left(\frac{W - \Delta F}{k_B T}\right) = \exp\left(\frac{W_{\text{dis}}}{k_B T}\right) \quad . \tag{12}$$

Simple manipulation of this ratio and integration of one side of the relation from $-\infty$ to ∞ gives,

$$\int_{-\infty}^{\infty} P_F(W) \exp\left(-\frac{W}{k_B T}\right) = \exp\left(-\frac{\Delta F}{k_B T}\right) \quad . \tag{13}$$

This is the content of the Jarzynski equality originally derived in [7] for Hamiltonian dynamics (see the footnote 4). In what follows, if not stated otherwise, we will only consider the forward process in a non-equilibrium experiment and drop the subscript F for the work probability distribution P_F. We will use the symbol $\overline{(...)}$

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[8]Non-equilibrium aging states are widespread in condensed matter physics. The most common example are structural glasses quenched below their glass transition temperature. The aging state is characterized by strong violations of the fluctuation-dissipation theorem [21].
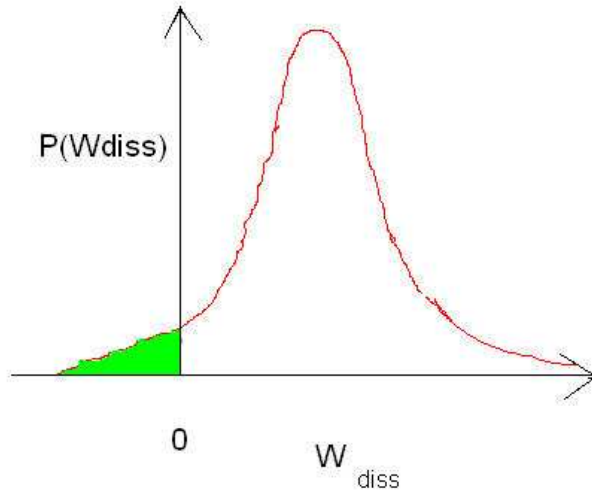
Figure 2: A typical work probability distribution for a small system is useful to characterize work fluctuations. Transient violations of the second law are a particular class of trajectories with work values characterized by the fact that the the Clausius inequality (2,3) is reversed.

to denote the average over all non-equilibrium trajectories generated by a given protocol. The Jarzynski equality (JE) reads,

$$\overline{\exp\left(-\frac{W}{k_B T}\right)} = \int_{-\infty}^{\infty} P(W)\exp\left(-\frac{W}{k_B T}\right) = \exp\left(-\frac{\Delta F}{k_B T}\right) \quad . \tag{14}$$

This expression can be written in a more compact form,

$$\overline{\exp\left(-\frac{W_{\mathrm{dis}}}{k_B T}\right)} = 1 \quad . \tag{15}$$

The Jarzynski equality provides a simple way to derive the second law. Using Jensen's inequality [24] $\langle \exp(x) \rangle \geq \exp(\langle x \rangle)$ in (15) we obtain,

$$\overline{W_{\mathrm{dis}}} \geq 0 \quad . \tag{16}$$

An important aspect of the JE (15) is that it introduces a way to quantitatively estimate transient violations of the second law, i.e. the fraction of trajectories whose dissipated work is negative. The reason is easy to understand by inspection

of (15). The average of the exponential in the l.h.s of (15) equals 1 only if trajectories with $\overline{W_{\mathrm{diss}}} < 0$ exist. An analysis of the JE in the near-equilibrium regime is useful. Close to equilibrium the distribution $P(W)$ can be approximated by a Gaussian [9],

$$P(W) = \frac{1}{\sqrt{2\pi\sigma^2}} \exp\left(-\frac{(W - W_{\mathrm{mean}})^2}{2\sigma^2}\right) \tag{17}$$

where W_{mean} is the value of the work at the center of the Gaussian and $\sigma^2 = \overline{W^2} - \overline{W}^2$ its variance. Substitution of (17) into (14) gives,

$$R = \frac{\sigma^2}{2\overline{W_{\mathrm{dis}}}k_BT} = 1 \quad . \tag{18}$$

Throughout the paper we will refer to R as the *fluctuation-dissipation ratio* as it involves a ratio between work fluctuations σ_2 and dissipation $\overline{W_{\mathrm{dis}}}$ [10]. This result is a particular form of the fluctuation-dissipation theorem in the linear response regime. In general, $R \neq 1$ far from equilibrium. More about the dependence of R with the value of the dissipated work will be said later in Sec. 7. We infer from (18) that in the near-equilibrium regime the variance of the work is of the same order of the average dissipated work. In the reversible limit $W_{\mathrm{dis}} \to 0$ the JE trivially holds as $W_{\mathrm{dis}} = 0$ (or $W = \Delta F$). In this limit the number of trajectories with $W_{\mathrm{dis}} > 0$ equals the number with $W_{\mathrm{dis}} < 0$, therefore the reversible limit is the case where the fraction of violating trajectories is maximal. This may look rather unexpected as transient violations might be thought to be a characteristic of non-equilibrium processes.

Transient violations of the second law are expected to decrease fast as the average value of the dissipated work $\overline{W_{\mathrm{dis}}}$ increases (for instance, if the size of the system increases) becoming unobservable in the thermodynamic limit. How transient violations are suppressed as the system size increases can be understood also from the JE. We rewrite (15) as,

$$1 = \overline{\exp\left(-\frac{W_{\mathrm{dis}}}{k_BT}\right)} = P_+ \left[\exp\left(-\frac{W_{\mathrm{dis}}}{k_BT}\right)\right]_+ + P_- \left[\exp\left(-\frac{W_{\mathrm{dis}}}{k_BT}\right)\right]_- \tag{19}$$

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[9]The exact form of the $P(W)$ can be very complicated, however the Gaussian approximation is expected to hold in the non-equilibrium regime where work trajectories do not deviate much from the reversible one. In this way the Gaussian approximation appears tightly related to linear response theory. In the latter, quantities deviate from their equilibrium values proportionally to the intensity of the perturbation. In a similar way, the deviation of the average work value (the first moment of $P(W)$) from its reversible value ΔF is expected to be linear with the perturbing speed $\dot{\lambda}$, see also [25] for a quantitative estimate of this statement. In other special cases, however, the Gaussian result can be exact even for arbitrarily strong perturbations. This is the example of a bead dragged through water [26].

[10]The use of the term *fluctuation-dissipation ratio* for the quantity R has not to be confused with that adopted in glassy systems and usually denoted by x [21]. For glassy systems x describes the ratio between a time derivative of the correlation function and the response function. Albeit similar, the two-quantities are not identical as they refer to different non-equilibrium scenarios.

where P_+, P_- are the probabilities to generate a trajectory with $W_{\text{diss}} > 0, W_{\text{dis}} < 0$ respectively, i.e. $P_+ + P_- = 1$. In analogous way, the square brackets $[..]_+, [..]_-$ denote averages $\overline{(..)}$ but restricted over the subsets of trajectories with $W_{\text{dis}} > 0, W_{\text{dis}} < 0$ respectively. In Fig. 2 P_+, P_- would correspond to the green and white areas under the distribution. Using this decomposition it is easy to understand how work trajectory values contribute to the r.h.s. of (19) enforcing the validity of the JE. The value of W_{dis} increases proportionally to the size N of the system (because the work is an extensive quantity). Because all four quantities appearing in the r.h.s. of (19) are positive, to impose a sum of both terms of 1, the factor p_- has to be exponentially small with the average value of the dissipated work (i.e. the size of the system) $p_- \sim \exp(-\mathcal{O}(N))$ to compensate the divergence of the corresponding average $[..]_-$. This implies $p_+ = 1 - \exp(-\mathcal{O}(N))$ so transient violations are exponentially suppressed with the system size, yet they have to be weighed for the JE to hold.

3.3   Free energy recovery from non-equilibrium experiments

An important consequence of the JE (14) is that non-equilibrium experiments can be used to recover equilibrium free-energy differences [7],

$$\Delta F = -k_B T \log\left(\overline{\exp\left(-\frac{W}{k_B T}\right)}\right) \quad . \tag{20}$$

The non-equilibrium work relation (20) is useful to find the equilibrium free-energy change along a given reaction when it is not possible to carry it out reversibly. The idea is to repeat non-equilibrium experiments many times and evaluate the exponential average in the r.h.s of (20) to derive the corresponding work in a reversible process. This formula has been used to recover the free-energy change in the folding-unfolding reaction for small RNA molecules, see Sec. 6 for a detailed exposition. However, there are practical difficulties in the applicability of (20) as the number of trajectories included in the exponential average must be actually infinite. This is unrealizable in practice as non-equilibrium experiments can be performed only a finite number of times and the finiteness of the number of trajectories introduces a bias. In what follows we will use ΔF_{JE} to denote the estimate for the equilibrium free energy ΔF obtained by using the JE given in (20) with a finite number of trajectories. As we remarked in the previous paragraph, the number of trajectories required to evaluate the JE grows exponentially with the average value of the dissipated work. The dependence of the bias and error with the number of pulls has been estimated in some cases [27, 28]. In general this dependence can be quite complicated as it depends on the behavior of the left tails of the distribution $P(W)$ which are difficult to analyze in general.

If the non-equilibrium experiment is done in the near-equilibrium regime then

it is better to use the fluctuation-dissipation (FD) estimate (18),

$$\Delta F_{FD} = \overline{W} - \frac{\sigma^2}{2k_B T} \quad . \tag{21}$$

In most cases it is difficult to determine whether the non-equilibrium process is done in the near equilibrium regime, so this estimate has to be taken with caution. A description of the bias and error for the estimates (20,21) in the near-equilibrium case has been recently given. Both are exact in the limit of infinite number of non-equilibrium trajectories. Interestingly, when the number of repeated experiments is small the JE estimate (20) provides a better estimate than the FD (21) does [28].

4   Experimental observation of work fluctuations

For sake of clarity we discuss now some examples where work fluctuations are experimentally measurable. Some of them have been already measured, others might be in the near future.

We start this tour discussing recent experiments on simple systems. This is the case of a micron-sized polystyrene bead confined in an optical trap and dragged through a solvent (e.g. water) of viscosity η at constant speed v. In average the viscous drag on the bead exerts a force γv that counteracts the force inside the trap $f(t) = -kx(t)$ where t denotes the time, k is the stiffness of the trap and $x(t)$ is the distance of the bead to the center of the trap. The control parameter is the position of the center of the trap $x_0(t)$ that moves at a constant speed $\dot{x}_0 = v$ and the fluctuating variable is the position $x(t)$ of the bead inside the trap (or equivalently the force $f(t)$ acting on the bead). The work along a trajectory of duration t_f is given by $W = \int_0^{t_f} ds f(s) v ds$. For such case work fluctuations were theoretically predicted [26] and recently measured [29].

Moving to more complex systems, recent experiments have studied the response   of biomolecules to mechanical force [30, 31, 32]. The advent of nanotechnologies has opened the possibility to exert very small forces on nanosized systems (from piconewtons $1pN = 10^{-12}N$ using optical or magnetic tweezers, to nanonewtons $1nN = 10^9N$ using AFMs). These techniques allow researchers to manipulate and study individual biomolecules one by one. Work fluctuations have been already observed in the unfolding of small RNA molecules (around 100 pair bases) under the action of mechanical force. More will be said below in Sec. 5. In these experiments the RNA molecule is held through linker polymers to two micron-sized beads. One is held by suction on the tip of a micropipette, the other is confined in the optical trap. The molecule is pulled as the distance $x(t)$ between the center of the optical trap and the tip of the micropipette increases at a given rate. $x(t)$ therefore defines the control parameter. The fluctuating variable in this case is the force $f(t)$ exerted on the whole system that is measured through the deflection of the bead in

the trap. The work along a given trajectory is again $W = \int_0^{t_f} f(s)\dot{x}(s)ds$ [11]. Work fluctuations are observed during the unfolding process due to the stochastic behavior of the breakage force at which the molecule unfolds. Similar experiments are expected to be conducted also for proteins [34], albeit the large molecular weight of such molecules might render the quantitative evaluation of work fluctuations difficult.

One might speculate also on the importance of work fluctuations on the behavior of molecular motors and their efficiency. In this case, work fluctuations are observable through single molecule experiments by measuring the mechanical force exerted upon the motor as it translocates along the template. Examples of these motors are DNA [38, 37] or RNA [35, 36, 39] polymerases during the replication and transcription process, helicases and topoisomerases that unwind the DNA [41] or the ribosome during the transcription process. Other cases include the condensation process of DNA inside the viral capside during the infection cycle [40], and gene regulatory mechanisms (such as transcription factors) ruled by protein-DNA interactions that expose large segments of condensed DNA to the replication machinery [42]. Work fluctuations are predicted to be observable in all these systems. Quantitative investigations will be surely conducted in the future.

Despite of their inherent interest, the measurement of work fluctuations in biomolecules has two important drawbacks: accuracy and reproducibility. Indeed, few single molecule experiments are fully reproducible due to the complexity of conditions and external factors required. Reproducibility at the single molecule level is specially serious in biomolecular processes requiring protein activity as many external factors strongly affect the outcome of the experiment. Accuracy is also an issue specially for measurements with nanometer resolution where stability and drift of the machines (e.g. optical tweezers) still impede high accuracy results.

Accurate and reproducible measurements of work fluctuations might be easier in systems with reduced complexity within the traditional domain of physics. One example is the already mentioned experiment of the bead in an optical trap moved through a solvent. High accuracy recent experimental measurements of the work between non-equilibrium steady-states confirm that such measurements are indeed possible [43]. Another example that has called our attention recently is the case of magnetic nanoparticle systems in a magnetic field [44]. Magnetic measurements in microsquids in Grenoble (France) have shown how it is possible to observe magnetization reversal of single magnetic nanoparticles of magnetic moment $\mu \sim 1000\mu_B$ (μ_B is the Bohr magneton) at low temperatures [45]. For magnetic nanoparticle systems the control parameter is the external magnetic field that can be switched at a constant speed (ramping experiments). The fluctuating or stochastic variable is the value of the field at which the magnetization reverses. The work along a given trajectory is then given by $W = -\mu \int_0^{t_f} M(t)\dot{H}(t)dt$. The aspect that makes these systems specially interesting is the possibility to use SQUID quantum (i.e.

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[11]In these experiments usually $x(t)$ is the distance measured from the center of the bead in the trap (rather than the center of the trap) to the center of the bead in the micropipette. This introduces a correction to the work that is negligible in most cases [33].

high-precision) technology to measure the magnetic moment in favor of a higher accuracy. Reproducibility is also easier to achieve as many physical properties of nanoparticles can be externally tuned, for example the height of the activation barrier and consequently the relaxation time of the nanoparticle as well. Other specific properties of magnetic nanoparticle systems makes them specially suitable to measure work fluctuations [44]. We may see these experiments done in the near future.

4.1 Measurement of heat fluctuations

Up to now we discussed about work fluctuations but nothing was said about heat fluctuations. The reason is simple. Work is much easier to measure than heat. Although transferred heat can be measured by using a small thermometer probe (by recording its change of temperature) heat fluctuations are another matter. The easiest procedure to measure heat along a trajectory is to use the first law of thermodynamics where $\Delta Q = \Delta E - W$. Knowledge of both the work and the energy change along a trajectory immediately gives the heat exchanged between system and the bath. Measuring the energy content of the system can be hopeless in many cases. Only in some special cases this is possible. Here I discuss two possible situations.

The first one corresponds to the case where no energy change occurs between the initial and final configuration for all non-equilibrium trajectories. This situation is realized in the magnetic example [44] discussed in the previous section where the reversal symmetry of the system under a field induces a zero energy change $E_f = E_i = -\mu H_0$ if the field is changed from $-H_0$ to H_0 in a ramping experiment (here we assume H_0 to be large enough for the initial and final magnetization to align in the direction of the field). In general, $\Delta E = 0$ can be accomplished in any non-equilibrium cycle assuming that the initial and final states are identical. In the case of the unfolding of the RNA molecule under applied force this can be achieved by considering non-equilibrium trajectories where the molecule first unfolds and then refolds along a given cycle.

The second situation corresponds to the case where, due to the inherent simplicity of the system, the energy is known. A relevant example is the particle confined in an optical trap. In that case the energy of the bead in the trap is well approximated by $E = (1/2)kx^2$ and therefore the value of ΔE is known for each trajectory. The distribution of exchanged heat shows interesting features as compared to the work that have been recently discussed by Zohn and Cohen [46].

5 Single molecule experiments

The advent of nanotechnologies has provided instruments and tools for scientists to manipulate individual molecules and follow their dynamical trajectories as they carry out specialized molecular tasks [47]. The research of molecular reactions
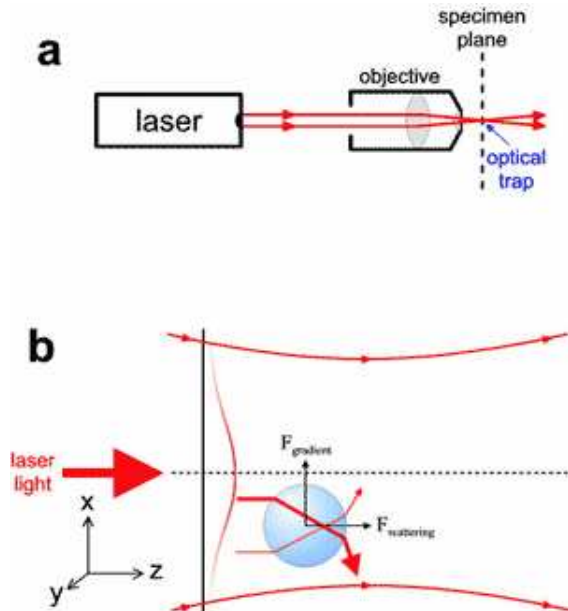
Figure 3: A single laser tweezers setup. (a) The laser light is focused into a spot by using an objective. (b) A Gaussian profile of light intensity generates a confining potential due to conservation of light momentum. The trapping force is induced by the difference in the index of refraction between the polystyrene bead and the surrounding water.

performed by individual molecules offers new insight on the importance of fluctuations and stochasticity in small systems. Mechanical force has been recognized as essential to understand the fate of many chemical reactions [48, 49]. Several force-microscopies are currently available to investigate the individual behavior of biomolecular complexes. Atomic force microscopy (AFM), optical and magnetic tweezers tweezers have become common tools that allow scientists to measure the response of these systems to applied external force. These techniques cover different but overlapping ranges of forces: AFM covers a range of forces spanning from several tens of pN up to hundreds of pN, optical tweezers span the intermediate region between 1pN and 100pN and magnetic tweezers are sensitive to tenths of pN.

Thermal fluctuations are important whenever the energies involved in molecular processes are of the order of several $k_B T$. A quick estimate of the forces participating in this regime can be obtained as follows. The typical distance d involving conformational changes at the biomolecular level is of the order of 1nm [12].

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[12]This is only a rough estimate, for instance the base pair distance in DNA is around one third

At room temperature $T = 298K$, $Fd = k_B T$ this gives a force $F \simeq 4pN$. Optical tweezers are ideal to investigate a large region of intermediate forces around this value [51]. Nowadays, optical tweezers are used to investigate many processes operated by biomolecules, ranging from the elastic deformation of nucleic acids or proteins to the specific action of enzymes acting on molecular substrates [50]. A typical experimental setup is shown in Fig. 3. Optical tweezers use light momentum conservation to generate a force gradient on polystyrene beads (of a diameter between 1 and 3 microns) that are immersed in water. Light deflection inside the beads arises from the difference in the index of refraction between the beads and water. In this way a confining potential can be generated by focusing a beam of light inside the chamber. To a high degree the confining potential can be considered as harmonic. Single beam tweezers can generate confining forces of the order of several tens of pN [13]. Dual tweezers use two counter propagating beams to generate higher forces (up to 150 pN) and have the advantage (by measuring the total amount of deflected light) that recurrent calibration is not required to measure forces. A fluid chamber is fixed in a movable stage or frame that is controlled by a piezo actuator. The chamber is made out of two parallel glass plates separated by a thin layer of parafilm. Inside the chamber there is a glass micropipette that can trap beads of the size of the micron by air suction. The two counter propagating laser beams can confine another bead in the optical trap. To measure forces on molecules a tether is attached to the two beads (one in the micropipette, the other in the trap). Attachments are designed by chemical treatment of the surface of the beads and chemical modification of the ends of the molecule (called labeling). As the stage is moved the force on the bead in the trap (and therefore, on the tether) can be measured. The distance between beads is then measured by using a light lever and a force-extension curve (FEC) can be recorded. Optical tweezers have been used in different fields ranging from physics to biology. A survey of their applications can be found in [50].

DNA plays a central role in biophysics [52, 53]. Accordingly its mechanical properties have been extensively investigated during the past 10 years [54, 55]. Initial investigations on the elastic response of double-stranded DNA under tension [56] have revealed that DNA behaves like an entropic spring as predicted by the worm-like chain model of polymer theory [57]. However, at difference with other polymers DNA shows structural transitions at modest forces (around or below 100pN) depending on how the molecule is pulled. For example, torsionally unconstrained double-stranded DNA shows a highly cooperative overstretching transition around 65pN [58, 59]. At the origin of this behavior there is the double-helix structure of DNA and the associated uncoiling of the two strands. However, if both strands are pulled from the same end of the DNA molecule,

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of a nanometer. This is the minimal distance that polymerases have to cover to elongate one base pair the newly synthesized strand.

[13] The confining force depends on wavelength of the light. Typical wavelength values are in the range 700-1000nm, lower frequencies are inadvisable as they can lead to light absorption and subsequent heat convection effects around the bead.
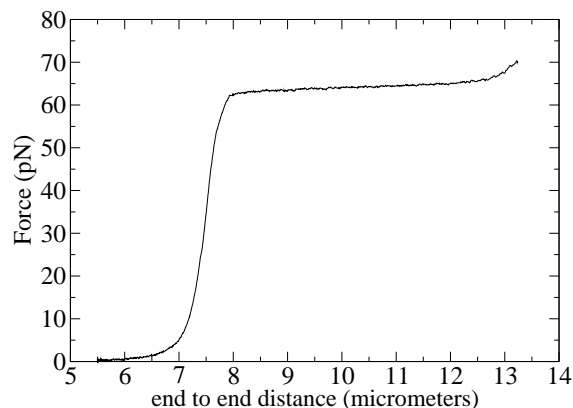
Figure 4: Force extension curve for a torsionally unconstrained DNA molecule of the λ bacteriophage, 24000 base pairs long in a water buffer at 100mM NaCl concentration and 7pH. The molecule has a contour length of approximately $8\mu m$ and shows the characteristic overstretching transition around 65 pN.

then DNA sequentially unzips at constant force following a curve that depends on the particular nucleotide sequence [60, 61]. The elastic response of DNA has produced many experimental [62, 63, 64, 65, 66] as well as theoretical investigations [67, 68, 69, 70, 71, 72] to characterize its structural transitions. A particular force-extension curve (FEC) showing the characteristic overstretching transition of double-stranded DNA is shown in Fig. 4.

6   Pulling experiments on RNA

RNA is an essential molecule in biochemistry. It plays an intermediate role between DNA (which encodes the genetic information and represents the "software" in living organisms) and proteins (which perform specialized tasks inside the cells and represent the "hardware"). Such intermediate role has been emphasized after the discovery that certain RNA molecules (called ribozymes) have catalytic activities that are essential in many regulational processes [73]. The relevance of RNA has motivated many single molecule studies. Compared to DNA, optical tweezers measurements in RNA present additional difficulties to the experimentalist. Not only RNA requires more elaboration in the synthesis of the molecular constructs, it is also a molecule very sensitive to the surrounding environment and degrades easier. Moreover, RNA domains have extensions of few tens of nanometers after unfolding, thus requiring more careful and precise measurements.

Liphardt et al. [74] have pulled RNA molecules and studied their unfolding by applying external force using optical tweezers. The molecular construct consists
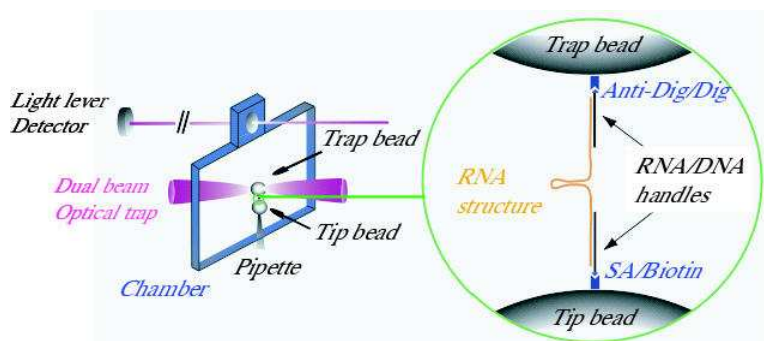
Figure 5: Typical experimental setup when pulling RNA molecules. The molecular construct consists of an RNA molecule attached by its ends to two RNA/DNA hybrid handles (to avoid formation of secondary structures in the handles). As compared to DNA, RNA single molecule experiments present additional difficulties as RNA quickly degrades and the resolution required to observe the unravelling of the molecule is much higher and of the order of the nanometer.

of two hybrid DNA-RNA handles that are annealed to the ends of a small RNA molecule, Fig 5. As the molecular construct is pulled the force-extension curve (FEC) reflects the elastic behavior of the handles (well described by a worm-like chain model [57]) until a force is reached where the molecule unfolds and a jump in the force and distance is observed.

Very interesting dynamical effects were later observed in small RNA hairpins depending on the pulling rate. For slow pulling rates the molecule was seen to follow always the same trajectory and unfold at a reproducible value of the critical force [14]. At this force coexistence and hopping between the folded and unfolded conformations has been observed characteristic of cooperative unfolding [74] [15]. More interesting, as the pulling rate increases larger hysteresis and stochastic fluctuations in the value of the breakage force were observed. Typically the average value of the breakage force tends to increase with the pulling rate. This dependence has been investigated by Evans and Ritchie [76, 77] who have applied Kramers theory [78] to describe the activated dynamics of a particle jumping over a force-dependent barrier as described by Bell [79]. The study of the loading rate dependence of the breakage force in this type of systems has led to new developments in what is now commonly referred as single-molecule force spectroscopy [80, 81], a technique that is useful to investigate the energy landscape of molecular

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[14]Reproducibility of trajectories has always limitations imposed by the unavoidable drift of the optical tweezers machine, see the remark at the end of Sec. 4. The accuracy in the value of the breakage force can be well controlled.

[15]The dependence of the value of the transition force and the hopping frequency on the sequence of the RNA molecule has been studied in [75]
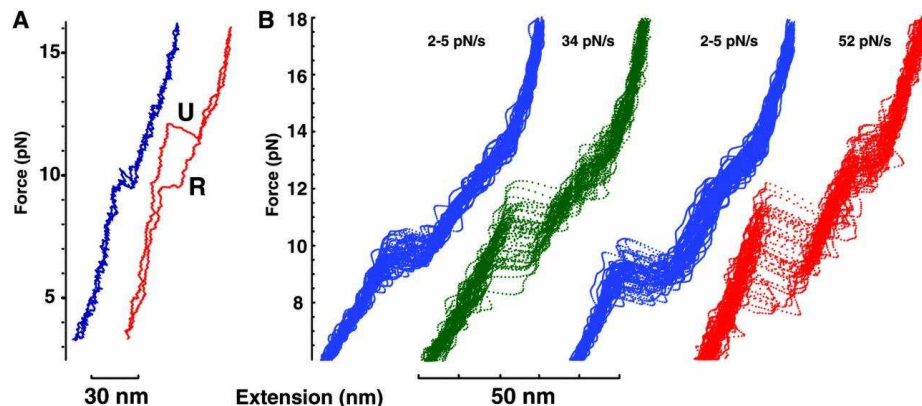
Figure 6: Non-equilibrium pulls in the RNA molecule P5abc at different pulling speeds in a buffer in the absence of magnesium. Panel A shows a reversible (left blue, pulling speed equal to 3-4pN/s) trajectory and an irreversible trajectory (right red, pulling speed 52pN/s). Panel B shows unfolding trajectories for two pulling speeds, 34pN/s (green) and 52pN/s(red) compared to near-equilibrium pulls at 2-5pN/s (blue). Figure taken from [74].

interactions. Typical unfolding curves showing the pulling rate dependence of the breakage force and the resulting hysteresis effects are shown in Fig. 6.

Hummer and Szabo have realized [82] that the non-equilibrium work relation (20) can be used in single molecule experiments to reconstruct the free energy landscape along the force coordinate. The JE (20) has been experimentally tested in [83] for the P5abc hairpin by repeated measurements of the work done along the unfolding trajectory at different pulling speeds. For P5abc in EDTA buffer the unfolding free energy change is well known from its secondary structure and therefore is a useful example to test the validity of the JE. Typical work histograms are shown in Fig. 7 for three pulling speeds. As expected, as the pulling rate increases the average value of the dissipated work increases reaching values of the order of $4k_BT$ at the fastest pulling speeds. The main result in [83] is that the JE can be used to predict the free energy change for the folding-unfolding transition in the P5abc hairpin with a precision within $1k_BT$ using a modest number of pulls (around 100). Moreover, the JE provides a better estimate for the equilibrium free-energy change than the FD estimate does (21). The advantage of the former as compared to the later has been verified in the near-equilibrium regime (where (18) holds), when the number of repeated pulls is not too large [28].

For the case of the P5abc in EDTA buffer the value of the dissipated work is small [16]. In general, for larger molecules the JE is expected to give less reliable

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[16]In this buffer conditions kinetic barriers are low. High kinetic barriers and strong irreversibil-
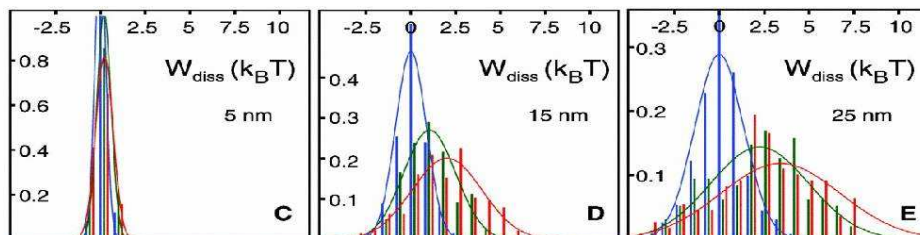
Figure 7: Work distributions for the P5abc molecule at different pulling speeds (3-5pN/s blue, 34pN/s green, 52pN/s red) measured at different distances along the pulling process. Figure taken from [83].

estimates for the equilibrium free-energy as the value of the average dissipated work increases. An example is shown in Fig. 8. Typical unfolding curves for a three way RNA junction are shown in Fig. 8. The validity of the JE for such cases is currently investigated [85]. Other more complex cases imply the unfolding of even larger RNA molecules consisting of many domains such as the recently investigated L21 RNA ribozyme [84].

7 Modeling the experiment

In [25] a two-state model has been studied to justify the non-equilibrium experiments in [83]. The main goal was to confirm that indeed it is possible to obtain the equilibrium free energy (within an error equal to $1k_BT$) by using the JE with a limited number of pulls done in that experiment. Interestingly, with this model it is possible to go quite far and find out several results regarding the kinetics of the unfolding process. This allows to make also specific predictions about the kinetic dependence of the dissipated work that can be experimentally tested as well as quantitative statements about the validity of the JE for two-state systems. Moreover, it is possible to do explicit calculations for the work distribution $P(W)$ and, if desired, go beyond the Gaussian case (17). Two-state models provide phenomenological descriptions of systems that can exist in two different forms, therefore the following considerations are expected to be applicable to many systems beyond the folding-unfolding dynamics of RNA molecules. In fact, the two-state model has been shown to provide a good description of the folding-unfolding dynamics of small DNA or RNA hairpins that display strong cooperativity [86, 87] as well as structural transitions in polymers [88]. The model is represented in Fig. 9 where the two conformations (folded and unfolded) are separated by an intermediate

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ity are obtained either by going to faster pulling speeds (however, this is not easy to accomplish due to limited experimental capabilities) or in different buffer conditions. For the latter, high kinetic barriers and many intermediate states are obtained in the presence of divalent cations such as magnesium that establish specific tertiary contacts between some bases.
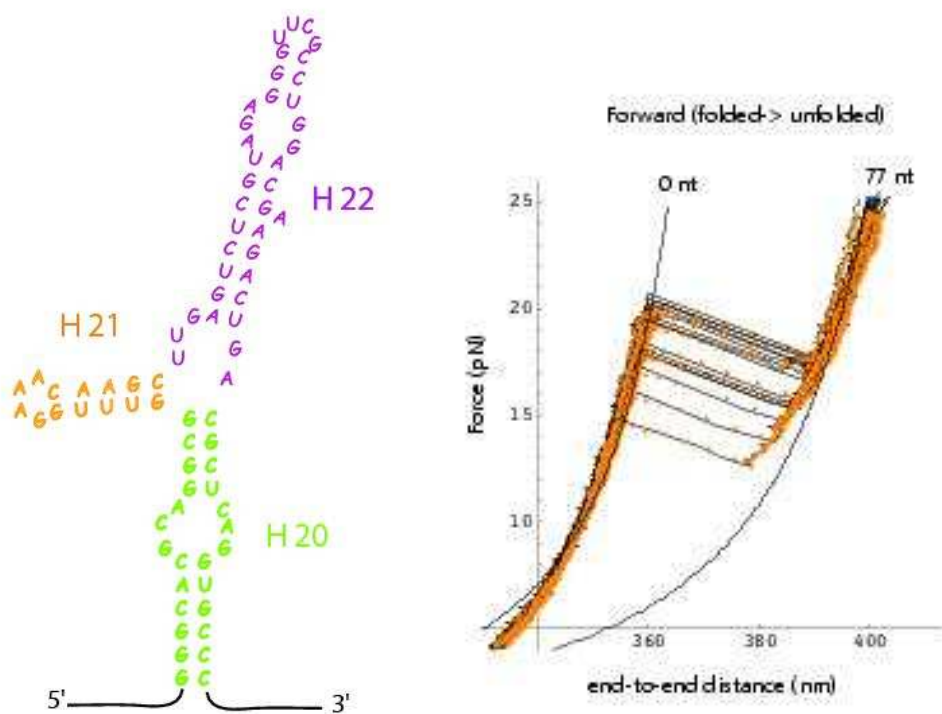
Figure 8: The left figure shows the structure of the junction in the 16S domain of the 30S ribosomal RNA subunit. The right figure shows some unfolding curves at 3.5pN/s. Courtesy of Delphine Collin.
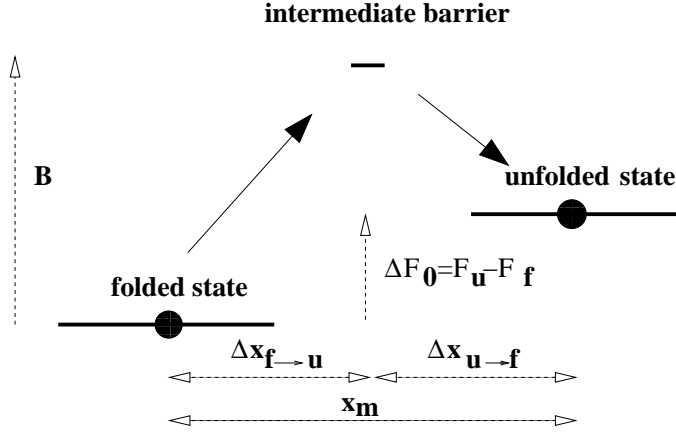
intermediate barrier

Figure 9: The two-state model with an intermediate barrier. The parameters are the free-energy gap ΔF_0, the unfolding distance x_m, the height B of the intermediate barrier and the distance of the intermediate barrier to the folded state $\Delta x_{f \to u} = x_m - \Delta x_{u \to f}$. Figure taken from [25].

barrier located at a distance $\Delta x_{f \to u}$ from the folded state and $\Delta x_{u \to f}$ from the unfolded state, the value $x_m = \Delta x_{f \to u} + \Delta x_{u \to f}$ being the total distance between the folded and the unfolded states. The free energy difference between the two states is denoted as ΔF_0 and the height of the barrier is indicated as B. Transition rates between the folded and the unfolded state are thermally activated and force dependent [79],

$$k_{f \to u}(f) = k_m k_0 \exp(-\beta(B - f\Delta x_{f \to u}))$$
$$k_{u \to f}(f) = k_m k_0 \exp(-\beta(B - \Delta F_0 + f\Delta x_{u \to f})) \tag{22}$$

where f is the external force, $\beta = 1/k_B T$, k_0 is a microscopic attempt frequency and k_m is a contribution arising from the handles, the bead in the trap and the machine [17]. The rates (22) satisfy detailed balance, a necessary condition for the equilibrium regime to be characterized by Boltzmann populations of the folded and unfolded states. The dynamics of the two-state model under the action of an external force has been analyzed in detail for the case of no-refolding process along the unfolding curve [76, 77], also called a first-order Markov process. This particular case is analytically tractable and specific predictions about the form of the work distribution can be made [89].

In the theoretical treatment of a non-equilibrium pulling experiment the force

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[17]In (22) we continue to use the term F for the Gibbs free energy. To be precise we should use instead G as for the experimental conditions the temperature and pressure of the bath are held constant.

can be taken as the control parameter [18] and increased at an approximately constant rate $r = \dot{f}$. The work exerted along a given trajectory is taken as $W = \int x df$. The probability distribution cannot be exactly evaluated in closed form and only the moments of the distribution can be computed in a perturbative scheme where the average dissipated work is assumed to be several times $k_B T$. The results have been given in [25] for the first two moments. These give the average dissipated work and its variance, from which the value of the fluctuation-dissipation ratio R can be inferred. The first two moments are the most relevant quantities as they can be directly compared with the experimental results. A general result for the average dissipated work can be derived in the linear-response regime where the pulling speed is slow compared to the hopping frequency at the transition force f_t (i.e. the value of the force at which the folded and unfolded populations of the RNA molecules are equal in equilibrium). The linear-response regime is therefore characterized by the dimensionless parameter ρ defined as,

$$\rho = \frac{r}{f_t k_{\text{total}}(f_t)} \tag{23}$$

where $k_{\text{total}}(f_t) = k_{u \to f}(f_t) + k_{f \to u}(f_t)$ is the total rate at the transition force. When $\rho < 1$ the average dissipated work is given by,

$$\overline{W_{\text{dis}}} \sim \rho \Delta F_0 + \mathcal{O}(\rho^2) \tag{24}$$

The linear dependence of (24) can be used to derive estimates for the relaxation time of the molecule that might complement other type of kinetic measurements (such as the measurement the folding-unfolding hopping frequency right at the transition force). In Fig. 10 we show the results for these quantities as a function of the pulling rate using some kinetic parameters in (22) to fit the experimental data. The dashed line in the left panel of Fig. 10 shows the linear response prediction (24). Note that the experimental points fall off the linear response curve, showing that the pulling rates investigated in [84] explore the far from equilibrium regime. This is an important result because it shows that, despite of the smallness of the value of the average dissipated work (in the range $2 - 4 k_B T$) the experiments were carried out far from equilibrium reinforcing the validity of the Jarzynski relation in such regime [19]. This conclusion is substantiated by the dependence of

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[18]Strictly speaking this is not true. As remarked in Sec. 4 the control parameter in pulling experiments using optical tweezers is not the force but the distance between the center of the optical trap and the tip of the micropipette. The pulling speed r is always an average value of the force-dependent speed along the unfolding curve. Under this approximation (which typically introduces a small correction), using the force or the distance as the control parameter turns out to be equivalent as $d(fx) = f dx + x df$, see footnote (**) in [25] for a related remark.

[19]Indeed, had the experiments been carried out in the near-equilibrium regime, then the recovery of the equilibrium free-energy change using the JE would be expected due to the smallness of the average values of the dissipated work. The fact that the value of the dissipated work is small, yet the system is far from equilibrium, is consequence of the smallness of the RNA molecule.
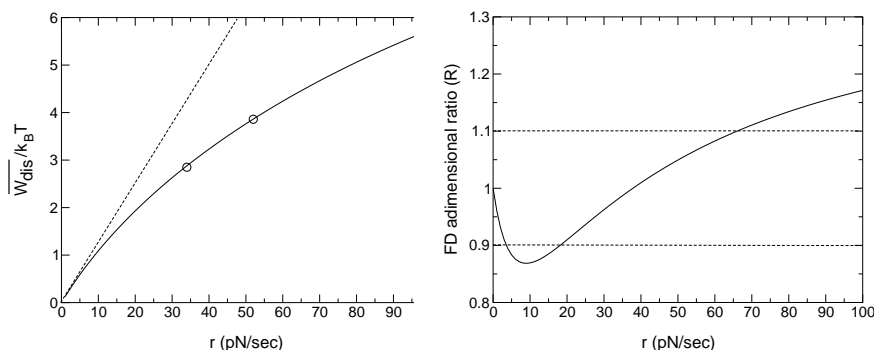
Figure 10: Average dissipated work (left) and fluctuation-dissipation ratio R (right) as a function of the pulling speed. The circles in the left figure are the experimental values. The values of the kinetic parameters characterizing the rates (22) have been chosen to fit the experimental data. The dashed line in the left figure shows the linear-response formula (24). The two horizontal dashed lines in the right figure limit a region of pulling speeds where the FD estimate (21) is expected to approximate well the free-energy change during the folding-unfolding reaction. Figure taken from [25].

the fluctuation-dissipation ratio (right panel in Fig. 10) which shows a strong non-monotonic behavior for pulling speeds above 20pN/s. Further evidence endorsing the fact that experiments were carried out far from the equilibrium regime is inferred from the shape of the work probability distributions $P(W)$ [20]. The results are shown in the left panel of Fig. 11 and were obtained from numerical simulations of the model using the kinetic values of the fitting parameters as derived from Fig. 10. Gaussian behavior is a fingerprint of the near-equilibrium regime, see the discussion in the paragraph containing footnote 9 in Sec. 3.2. As a comparison we show in the right panel of Fig. 11 the histograms obtained from the experiments. Both theory and experiments bear a close resemblance. Fig. 11 reveals the existence of long tails at both sides of the work distribution that strongly deviate from the Gaussian behavior.

Finally we provide an answer to the original question with which we started this section. Can we support the main result of the experiment [83] where a small number of pulls (around 60) was enough to obtain the equilibrium free energy (within an error of $1k_BT$) by using the JE? In Fig. 12 we compare the bias error

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[20]It must be emphasized though that this statement would not be valid if, by some reason, the parameters used to fit the kinetic data were completely off from the actual values. Although far-fetched, this possibility cannot be ruled out as the two-state model here considered is probably a crude approximation to the real description of the unfolding process (see the discussion in Ref. [49]). New experiments in other RNA hairpins are required to reach a better understanding.
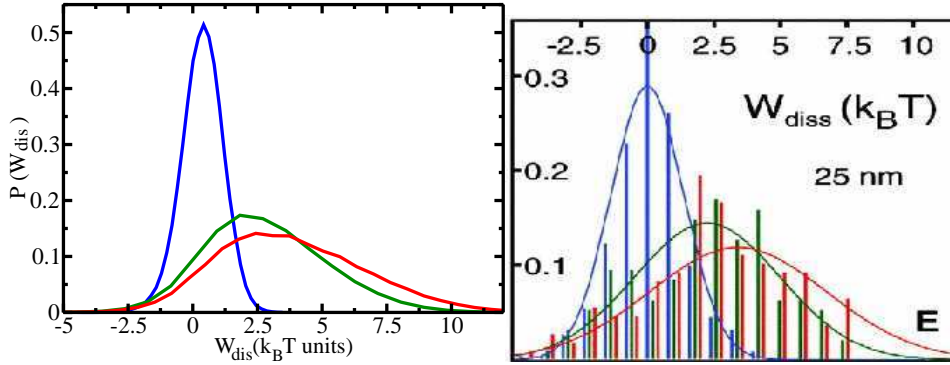
Figure 11: Work distributions obtained for the model (left) compared to the experimental results. Strong deviations from a Gaussian behavior are predicted, specially in the left tails of the distribution. Figures taken from [25, 83].

obtained from the two estimates (20,21) as well as from the average dissipated work $\overline{W}_{\text{dis}}$ along the force coordinate. The different bias errors are defined as,

$$B^{\text{dis}} = \overline{W} - \Delta F = \overline{W}_{\text{dis}} \tag{25}$$

$$B^{FD} = \Delta F_{FD} - \Delta F = \overline{W}_{\text{dis}}(1 - R) \tag{26}$$

$$B^{JE} = \Delta F_{JE} - \Delta F = -\log\left(\overline{\exp(-\frac{W_{\text{dis}}}{k_B T})}\right) \tag{27}$$

where (18) and (20,21) have been used. The bias error depends on the number of pulls N_{pulls}. To obtain these bias values we have averaged (25,26,27) over a large number of sets of experiments, each set characterized by N_{pulls} repeated pulls. Full convergence to the correct free-energy, as the number of pulls increases, corresponds to a vanishing bias throughout the force axis. From Fig. 12 we can see how the values obtained from the average dissipated work (25) and the FD estimate (26) quickly converge to limiting curves characterized by a finite bias. However, the bias obtained from the JE (27) slowly converges to zero and practically vanishes only for $N_{\text{pulls}} \sim 10^6$. Also, from the JE bias (27) shown in Fig. 12 we learn that 100 pulls are enough to get an estimate of the free-energy within $1k_B T$ of error for the folding-unfolding reaction, by using non-equilibrium work values at the two largest pulling speeds. We mention also that the FD estimate works well in the large force region of the force axis (around 20pN) but not in the intermediate force region (around 14pN) where it develops a bump. The reason why the FD works so well at high forces has it root in the behavior of the fluctuation-dissipation ratio R shown in the right panel in Fig. 10. There R has been evaluated from a pulling protocol where the force is ramped from 0 to a
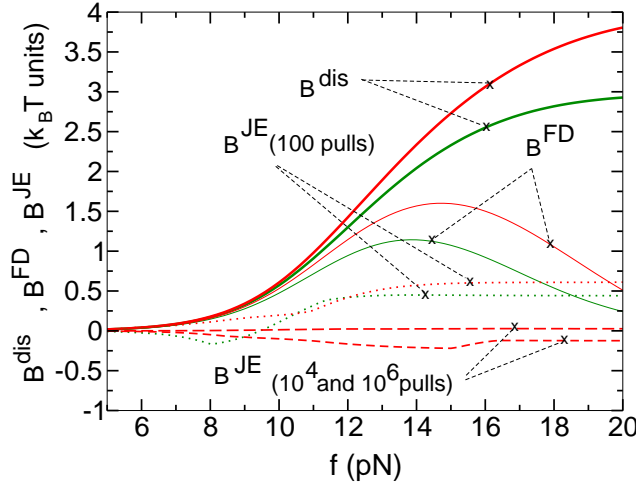
Figure 12: Behavior of the different bias defined in (25,26,27) along the force coordinate. Figure taken from [25].

value around 20pN. In that case R is close to 1 for a large region of pulling speeds (delimited by the two horizontal dashed lines). Whenever $R \sim 1$ the FD estimate is expected to work well if the number of pulls is not too small.

The number of pulls required to obtain the equilibrium free energy with an error within $1 k_B T$ can be estimated by measuring B^{JE} averaged over many sets, each one containing N_{pulls} repeated pulls. The dependence of the bias B^{JE} with N_{pulls} is shown in Fig. 13. The decay of the bias with N_{pulls} can be very well approximated by a power law $(N_{\text{pulls}})^{-\alpha(r)}$ where the exponent $\alpha(r)$ depends on the pulling rate (or the average dissipated work as they are related each other). The bias B^{JE} shows as a crossover to a $1/N_{\text{pulls}}$ behavior for $N_{\text{pulls}} > 1000$ in agreement with the prediction by Wood [90]. For a Gaussian process in the near-equilibrium regime the value of the exponent $\alpha(r)$ has been estimated numerically [28] and is relatively close to the values found in this case. From Fig. 13 we see the number of pulls required for the bias B^{JE} to be equal to $1 k_B T$ (indicated as the horizontal dashed line). This number of pulls is then shown in the inset of Fig. 13 as a function of the average dissipated work (also the pulling speeds are indicated). Under certain assumptions (see [91]), and only for modest values of the average dissipated work [91], this number of pulls can be shown to approximately grow as $\exp(R_{-}\frac{\overline{W_{\text{dis}}}}{k_B T})$ with R_{-} a constant of order unity that characterizes the left side tail of the work distribution. For the experimental values of the pulling speed considered in the experiment (the region limited by the square box shown in the figure in the Inset) the required number of pulls to get the desired accuracy is of the order of several tens as done in the experiment.
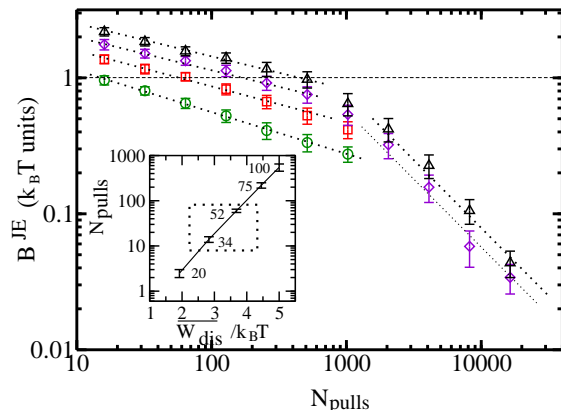
Figure 13: Main panel: Bias error (in units of k_BT) for the Jarzynski average (27) as function of the number of pulls for different pulling rates (from bottom to top: 34 (green),52 (red),75 (violet),100 (black) pN/s). Data have been averaged over 1000 sets and error bars correspond to 100 sets. Inset: Number of pulls necessary to obtain an estimate for the equilibrium free energy within k_BT and fit to the estimate $N_{\text{pulls}} \sim \exp(R_- \frac{\overline{W_{\text{dis}}}}{k_BT})$ which yields $R_- \simeq 1.5$.

All in all, the two-state model reproduces quantitatively many aspects of the non-equilibrium behavior observed in the experiment [84] and justifies the test of the validity of the JE there claimed.

8   Conclusions

Thermodynamics represented a great step in the development of science. It provided a general framework to understand all natural processes that involve the transformation of different sorts of energy (mechanical, chemical, electromagnetic) into work and heat. While work can be viewed as useful energy, heat represents energy that is not useful. The second law of thermodynamics limits the amount of useful work that can be extracted from heat. As heat abounds in nature it seems plausible that the level of organization that we see today in the form of biological matter originates from certain properties that characterize heat exchange processes.

Statistical mechanics provided a mechanistic picture of the abstract concepts of thermodynamics in terms of the average behavior of a large number of atoms or molecules and their interactions. According to this picture, thermodynamic quantities are not strictly constant but fluctuate around their average values. However, the amount of these fluctuations is small relative to the value of the thermodynamic quantities themselves. Much larger fluctuations are hardly observable and

become irrelevant as the macroscopic level is approached.

Fluctuation theorems go beyond this statistical level of description by quantifying fluctuations arbitrarily large whose magnitude can be of the same order of the average value. This is the content of the non-equilibrium work relation originally derived by Jarzynski [21]. In that case, work trajectories quite far from the average or most probable trajectory, have to be properly weighed for the equality to be satisfied. As the system size increases (or as the time increases for steady state systems) the probability to observe these rare trajectories quickly decreases. Were we repeat many times the dynamical experiment, the time we should wait until finding a trajectory that notably reduces the bias error associated with the equality, increases exponentially with the size of the system, ultimately reaching values that are of the order of the Poincare recurrence time. We then considered the suggestive fact that most of the non-equilibrium trajectories that enforce the validity of the Jarzynski equality, are also those that inspired many of the paradoxes underlying the statistical interpretation of heat and that were proposed in the early days of statistical mechanics.

What is the fundamental value of these rare trajectories described by fluctuation-theorems? If fluctuation theorems were just theorems, the value would be predominantly academic. There is of course interest in using the non-equilibrium work relation to obtain free energies for transformations that cannot be carried out reversibly. However, it might be possible that fluctuation theorems have an added fundamental value. They could provide physicists with a tool to explore the validity of the principles underlying some energy transformation processes. In the same way that classical mechanics proved inadequate to describe energy exchange between radiation and matter at the atomistic level, one could imagine that current theories describing thermal exchange processes occurring at very small length-scales or short times should be accordingly revised. In fact, all fluctuation theorems use in one way or another the concept of microscopic reversibility. This condition ensures that systems thermalize if left to evolve for a long time. However, it might be possible that microscopic reversibility holds only in average, that transitions at the microscopic level have unexpected properties with important consequences for biology and life [22]. If this were the case, while the average behavior would be well described by current dynamical theories, rare fluctuations might display a more refined pattern beyond our current expectation.

Biological matter tends to organize reaching fantastic levels of complexity. Although it is often tacitly assumed that our current understanding of physics will provide clues to fill into the many "details" that surround the organization of biological matter, the truth is that bold ideas will be probably needed to go beyond

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[21]We did not mention in this feature extensions of the classical non-equilibrium work relation to the quantum regime. Although several papers have recently appeared in the literature [92, 93, 94, 95], the concept of a quantum trajectory and quantum work are to be clarified and the first experimental attempt to test the corresponding quantum relations is still to be done.

[22]Ideas such as *purposiveness* of changes have appeared recurrently in the context of natural selection in biology, see for instance [96].

the present state of the art. Biological matter will become a common laboratory for physicists in order to test and understand many of the questions that transcend the behavior of ordinary matter. Single molecule experiments have opened a vein of research for physicists, that require the combination of a general knowledge of physics, chemistry and biology to grasp the most relevant aspects required to unravel the behavior of living matter at the most fundamental level.

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Félix Ritort
Departament of Physics
Faculty of Physics
University of Barcelona
Diagonal 647
08028 Barcelona, Spain
and
Department of Physics
University of California
Berkeley CA 94720, USA
email : ritort@ffn.ub.es